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A Transition State Analogue Inhibitor Combinatorial Library

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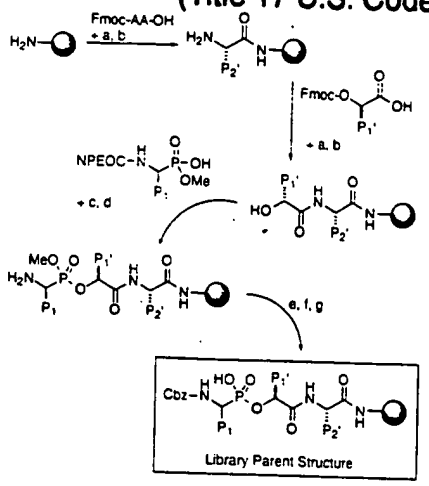
Combinatorial libraries are emerging as an integral part of the medicinal chemist's repertoire in the search for therapeutic agents.[1] Traditionally the development of a new drug begins with the identification of a lead compound generated from natural product collections or in-house chemical databases. Once a lead compound has been identified, medicinal chemists serially synthesize hundreds to thousands of individual variants of the original structure, with each variant submitted for biological testing to optimize *in vitro* and *in vivo* therapeutic efficacy. The ability to construct synthetic combinatorial libraries, which now include biopolymers,[2] nonnatural polymers,[3] and nonpolymeric organic compounds,[4] facilitates this process.

Our strategy has been to incorporate pharmacophores of proven therapeutic value into combinatorial libraries. Replacement of a peptide substrate's scissile carboxamide group with a phosphonic acid ester has yielded potent inhibitors of metalloproteinases,[5] including a number of orally active ACE inhibitors developed by the Squibb group.[6] We recently described an improved procedure for the synthesis of phosphonic acid esters[7] as well as the solid phase synthesis of peptidylphosphonates.[8] We now report the construction of a peptidylphosphonate combinatorial library and characterization of its interactions with thermolysin, a well-studied zinc endopeptidase from *Bacillus thermoproteolyticus*.

The split bead method[9] was used to construct a library of peptidylphosphonate sequences (Cbz-X^P-^{O}Y-Z-resin) on noncleavable resin (Figure 1).[10] The Z residue (P_2' position) consisted of 18 natural amino acids, excluding cysteine and asparagine.[11] Five α-hydroxy acids[12] were used at the ^{O}Y residue (P_1' position) and six α-aminoalkylphosphonic acids[13] at the X^P residue (P_1 position). For amino acid and α-hydroxy acid condensations, double couplings were performed, first with HOBt/HBTU and then with PyBroP. For phosphonic acid couplings, a modified Mitsunobu reaction (DIAD, tris(4-chlorophenyl)phosphine, DIEA) was used. The N-termini of the sequences were then capped with the Cbz group, and the library was deprotected with trifluoroacetic acid and appropriate scavengers. This resulted in the generation of 540 peptidylphosphonates (6 × 5 × 18) within the library.[14] The use of high reagent concentrations (≥100 mM), a large molar excess of reagents relative to resin-bound material (≥10 equiv), and longer reaction times resulted in yields routinely >90%.[15]



Total degeneracy: 6 (P_1) X 5 (P_1') X 18 (P_2') = 540

Figure 1. Solid phase peptidylphosphonate synthesis cycle. (a) HBTU, HOBt, DIEA, NMP (1×), PyBroP, DIEA, NMP (1×); (b) 30% peperidine/NMP; (c) tris(4-chlorophenyl)phosphine, DIAD, DIEA, THF; (d) 5% DBU/NMP; (e) Cbz-Cl, DIEA, dioxane; (f) 1:2:2 thiophenol–triethylamine–dioxane; (g) triethylsilane, TFA.

The peptidylphosphonate library was assayed for thermolysin inhibition while attached to the resin.[16] After rank ordering of each mixture with a depletion assay,[17] an iterative strategy for active sequence identification was employed.[18] Thus, after each round of screening, the most active pool was selected for deconvolution. Each subpool was then synthesized and assayed with the most active mixtures then chosen for the next round of screening.

The X^P residue library consisted of six mixtures of peptidylphosphonate sequences (Cbz-X^P-^{O}Y-Z-resin), with each mixture containing 90 compounds. While all of the mixtures interacted with thermolysin to some extent compared to the control (acetylated resin), a definite rank ordering was observed

(1) (a) Gallop, M. A.; Barrett, R. W.; Dower, W. J.; Fodor, S. P.; Gordon, E. M. *J. Med. Chem.* **1994**, *37*, 1233. (b) Gordon, E. M.; Barrett, R. W.; Dower, W. J.; Fodor, S. P.; Gallop, M. A. *J. Med. Chem.* **1994**, *37*, 1385.

(2) (a) Fodor, S. P.; Read, J. L.; Pirrung, M. C.; Stryer, L.; Lu, A. T.; Solas, D. *Science* **1991**, *251*, 767. (b) Lam, K. S.; Salmon, S. E.; Hersh, E. M.; Hruby, V. J.; Kazmierski, W. M.; Knapp, R. J. *Nature* **1991**, *354*, 82. (c) Houghten, R. A.; Pinilla, C.; Blondelles, S. E.; Appel, J. R.; Dooley, C. T.; Cuervo, J. H. *Nature* **1991**, *354*, 84. (d) Bock, L. C.; Griffin, L. C.; Latham, J. A.; Vermann, E. H.; Toole, J. J. *Nature* **1992**, *355*, 564.

(3) (a) Cho, C. Y.; Moran, E. J.; Cherry, S. R.; Stephans, J. C.; Fodor, S. P.; Adams, C. L.; Sundaram, A.; Jacobs, J. W.; Schultz, P. G. *Science* **1993**, *261*, 1303. (b) Zuckermann, R. N.; Martin, E. J.; Spellmeyer, D. C.; Stauber, G. B.; Shoemaker, K. R.; Kerr, J. M.; Figliozzi, G. M.; Goff, D. A.; Siani, M. A.; Simon, R. J.; Banville, S. C.; Brown, E. G.; Wang, L.; Richter, L. S.; Moos, W. H. *J. Med. Chem.* **1994**, *37*, 2678.

(4) (a) Bunin, B. A.; Ellman, J. A. *J. Am. Chem. Soc.* **1992**, *114*, 10997. (b) DeWitt, S. H.; Kiely, J. S.; Stankovic, C. J.; Schroeder, M. C.; Cody, D. M.; Pavia, M. R. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 6909.

(5) (a) Bartlett, P. A.; Marlowe, C. K. *Biochemistry* **1987**, *26*, 8553. (b) Thorsett, E. D.; Harris, E. E.; Peterson, E. R.; Greenlee, W. J.; Patchett, A. A.; Ulm, E. H.; Vassil, T. C. *Proc. Natl. Acad. Sci. U.S.A.* **1982**, *79*, 2176.

(6) Karanewsky, D. S.; Badia, M. C.; Cushman, S. W.; DeForrest, J. M.; Dejneka, T.; Loots, M. J.; Perri, M. G.; Petrillo, E. W.; Powell, J. R. *J. Med. Chem.* **1988**, *31*, 204.

(7) (a) Campbell, D. A. *J. Org. Chem.* **1992**, *57*, 6331. (b) Campbell, D. A.; Bermak, J. C. *J. Org. Chem.* **1994**, *59*, 658.

(8) Campbell, D. A.; Bermak, J. C. *J. Am. Chem. Soc.* **1994**, *116*, 6039.

(9) Furka, A.; Sebestyen, R.; Asgedom, M.; Dibo, G. *Int. J. Pept. Protein Res.* **1991**, *37*, 487.

(10) TentaGel-S-NH_2 resin without a cleavable linker (90 μm, 230 μmol/g) was obtained from Rapp Polymere.

(11) Cysteine would necessitate the use of reducing agents during all deprotected library manipulations. Low coupling yields between H-Asn(Trt)-resin and the α-hydroxy acids were observed.

(12) The P_1' monomer basis set included glycolic acid, (*R*)-lactic acid, (*R*)-mandelic acid, 3(*R*)-phenyllactic acid, and 2(*R*)-hydroxyisocaproic acid.

(13) The P_1 monomer basis set included glycine, (*R,S*)-alanine, (*R,S*)-valine, (*R,S*)-leucine, (*R,S*)-isoleucine, and (*R,S*)-phenylalanine.

(14) A number of the phosphonic acid building blocks were racemic, and inclusion of diastereomeric sequences increases the number of peptidylphosphonates within the library to 900.

(15) As determined from the dibenzofulvene–piperidine absorbance at 302 nm with Fmoc-protected amino acids and α-hydroxy acids and 4-nitrostyrene absorbance at 308 nm with ((nitrophenyl)ethoxy)carbonyl (NPEOC)-protected α-aminoalkylphosphonic acids.[8]

(16) This circumvents library bias during postcleavage manipulations. For example, precipitation of a cleaved soluble library with ether to remove scavengers biases the library toward polar compounds since nonpolar sequences will have varying solubilities in the ether and may not completely precipitate.

(17) Library mixtures and thermolysin were incubated together and then filtered to remove resin-bound enzyme/inhibitor complex. Proteolytic activity of the filtrates was then assayed and used to rank order the library mixtures. A somewhat related procedure has been described previously: Barrett, R. W.; James, I. F.; Goldstein, A. *Biochem. Biophys. Res. Commun.* **1986**, *137*, 316.

(18) Blake, J.; Litzi-David, L. *Bioconjugate Chem.* **1992**, *3*, 510.

0002-7863/95/1517-5381$09.00/0

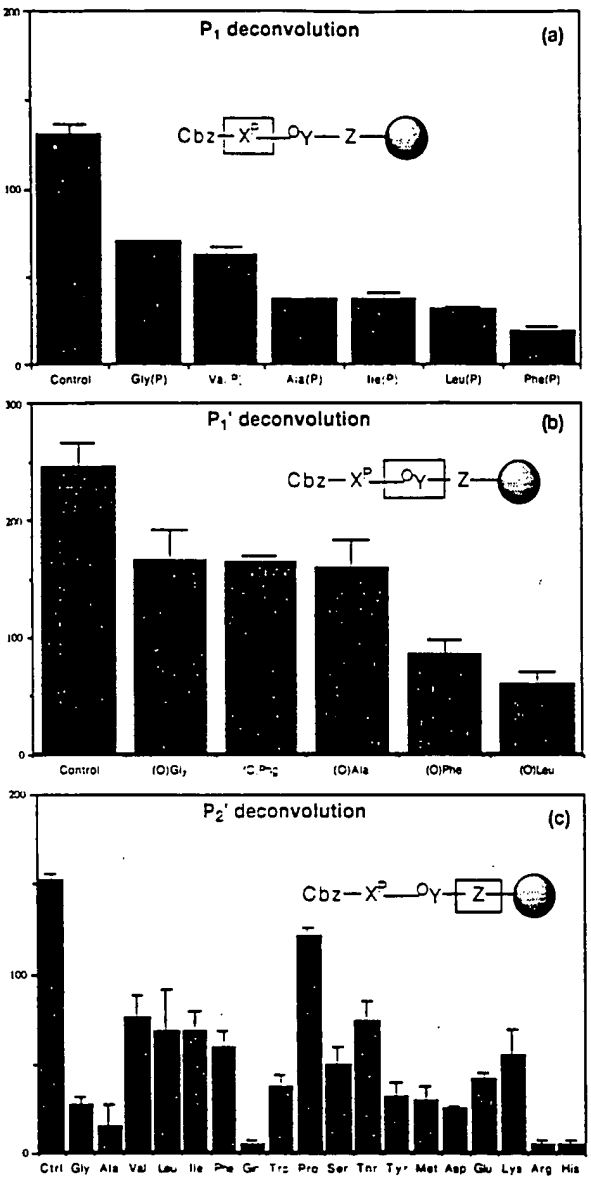


Figure 2. Depletion assay results. Potent peptidylphosphonate inhibitors of thermolysin were identified using an iterative strategy to determine the sequences of the most active library mixtures. The y-axis shows the hydrolysis rate (mA/min). Error bars indicate standard deviations.

(Figure 2a). The rank order indicated the following preference at the P_1 position: $Phe^P > Leu^P > Ile^P$, $Ala^P > Val^P > Gly^P$, which is in agreement[19] with a series of peptidylphosphonate inhibitors that have been described in the literature (Cbz-X^P-^{O}Leu-Ala(OH), where X = Phe, Leu, Ala, Gly have K_i = 45 nM, 680 nM, 1.8 μM, and 13 μM, respectively).[5a]

The ^{O}Y position library consisted of five mixtures, each containing 18 peptidylphosphonate sequences (Cbz-Phe^P-^{O}Y-Z-resin). The depletion assay indicated that the α-hydroxy acid analogue of leucine was the preferred P_1' residue (Figure 2b). Although a series of peptidylphosphonate inhibitors with varying P_1' residues has not been described in the literature, obviating direct comparisons with the ^{O}Y position library rank order, Bartlett has shown that a linear correlation exists between the K_i of a peptidylphosphonate inhibitor and k_{cat}/K_M of its corresponding peptide substrate.[5a] The reported k_{cat}/K_m ($s^{-1}\cdot mM^{-1}$) values of Cbz-Phe-Y-Ala(OH), where Y = Gly, Ala, Phe, and Leu, are 1.05, 8.4, 360, and 578, respectively, in agreement with the P_1' library rank order.[20]

The Z position library consisted of 18 individual peptidylphosphonate sequences (Cbz-Phe^P-^{O}Leu-Z-resin). This library contained a number of amino acids that exhibited a high affinity for thermolysin (Figure 2c). In addition to identifying the most potent peptidylphosphonate sequence for inhibition of thermolysin described in the literature (P_2' = Ala),[5a] this combinatorial strategy uncovered additional active sequences containing the basic amino acids arginine and histidine and the carboxamide side chain amino acid glutamine at P_2'.

Since the tethered library does not enable *à priori* determination of carboxy terminus preference, Cbz-(R,S)-Phe^P-^{O}Leu-Ala-X was synthesized as both the carboxylic acid **1** (X = OH) and the amide **2** (X = NH_2). The amide **2** was 2.5 times more potent than the carboxylic acid **1** (49 versus 122 nM).[21] Based on this observation, all remaining peptidylphosphonates were synthesized as amides. Sequences containing the basic side chains histidine **3** (K_i = 57 nM) and arginine **4** (K_i = 64 nM) at P_2' were equipotent to **2**, while slightly lower activity was obtained with glutamine at P_2' **5** (K_i = 127 nM).[22] *These represent novel inhibitors of thermolysin, and their discovery was unexpected since all the inhibitors that have been reported in the literature contain hydrophobic residues at the P_2' position.*[23]

This study emphasizes some important advantages of the combinatorial library approach to inhibitor discovery. As a consequence of the limited number of compounds a medicinal chemist can synthesize in a reasonable amount of time, the compounds chosen for synthesis are constrained by pre-existing knowledge about the system under study. As a result, the work takes place in a local minimum that probably constrains most competitors working in the field also. However, by judicious choice of the monomer basis set, combinatorial strategies investigate a far greater region of space and are more likely to discover configurations that are closer to the absolute local minima or exist in divergent local minima. An additional advantage is that besides identifying a lead compound(s), significant structure–activity data are generated which can then be used for *in vivo* activity optimization.

In summary, a peptidylphosphonate combinatorial library has been constructed and used to identify a number of potent thermolysin inhibitors. The most active peptidylphosphonates are Cbz-(R,S)-Phe^P-^{O}Leu-Arg(NH_2) (K_i = 64 nM), Cbz-(R,S)-Phe^P-^{O}Leu-His(NH_2) (K_i = 57 nM), and Cbz-(R,S)-Phe^P-^{O}Leu-Ala(NH_2) (K_i = 49 nM). The histidine and arginine residues at P_2' represent a significant deviation from thermolysin inhibitors previously described, which typically contain hydrophobic groups at that position. The strategy of constructing combinatorial libraries that incorporate a pharmacophore within a biopolymer to increase inhibitor potency, as well as to reduce the size of the inhibitors, has now been validated and should be applicable to other pharmacophores and enzymes.

Supplementary Material Available: Experimental procedures for construction of the peptidylphosphonate library, the deconvolution protocol, and the inhibitor K_i determination procedure (4 pages). This material is contained in many libraries on microfiche, immediately follows this article in the microfilm version of the journal, can be ordered from the ACS, and can be downloaded from the Internet; see any current masthead page for ordering information and Internet access instructions.

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(19) Assuming that library mixtures and single compounds exhibit similar binding profiles.

(20) Morihara, K.; Tsuzuki, H. *Eur. J. Biochem.* **1970**, *15*, 374.

(21) K_i values for both enantiomers of Cbz-Phe^P-^{O}Leu-Ala(OH) have been reported (45 nM and 30 μM for the *R* and *S* enantiomers, respectively).[5a] HPLC analysis indicated that the two diastereomers were present in approximately equal amounts, resulting in an estimated value of 61 nM for the *R* enantiomer.

(22) A cross section of peptidylphosphonate sequences that were less active in the depletion assay had significantly lower K_i values.

(23) Rich, D. H. In *Comprehensive Medicinal Chemistry*; Sammes, P. G., Ed.; Pergamon Press: New York, 1990; Vol. 2, p 391.